

RESEARCH PAPER

Ectopic divisions in vascular and ground tissues of *Arabidopsis thaliana* result in distinct leaf venation defects

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Abstract

Leaf venation patterns vary considerably between species and between leaves within a species. A mechanism based on canalization of auxin transport has been suggested as the means by which plastic yet organized venation patterns are generated. This study assessed the plasticity of *Arabidopsis thaliana* leaf venation in response to ectopic ground or procambial cell divisions and auxin transport inhibition (ATI). Ectopic ground cell divisions resulted in vascular fragments between major veins, whereas ectopic procambial cell divisions resulted in additional, abnormal vessels along major veins, with more severely perturbed lines forming incomplete secondary and higher-order venation. These responses imply limited vascular plasticity in response to unscheduled cell divisions. Surprisingly, a combination of ectopic ground cell divisions and ATI resulted in massive vascular overgrowth. It is hypothesized that the vascular overproduction in auxin transport-inhibited wild-type leaves is limited by simultaneous differentiation of ground cells into mesophyll cells. Ectopic ground cell divisions may negate this effect by providing undifferentiated ground cells that respond to accumulated auxin by differentiation into vascular cells.

Key words: Auxin, BCTV-C4, ectopic cell division, ground tissue, leaf venation, procambium.

Introduction

Plant vascular systems consist of a continuous network of interconnected cells throughout the plant, showing organ- and species-specific architectures. While considerable research effort has examined the genetic control of vascular differentiation and several factors are known to affect vascular cell proliferation (reviewed in Mähönen *et al.*, 2000; Dettmer *et al.*, 2009; Caño-Delgado *et al.*, 2010; Ohashi-Ito and Fukuda, 2010), the role of controlled cell divisions in this process is not clear. Procambial cells are derived from patterned cell divisions within the ground tissues, and procambial and ground cell tissue types affect vascular strand formation (Scarpella *et al.*, 2004; Kang *et al.*, 2007). How these two adjacent tissues interact would seem likely, therefore, to affect vascular patterning.

A classic paper published more than 50 years ago proposed that auxin can induce differentiation of vascular cells along paths of active polar auxin transport (PAT; Jacobs, 1952). Numerous subsequent publications have confirmed and expanded on the key role of auxin and PAT in vascular patterning and differentiation (reviewed in Fukuda, 2004). According to the canalization of auxin flow hypothesis (Sachs, 1981), diffuse sources of auxin are gradually channelled into narrow file(s) of cells to induce discrete vascular bundles. The process of auxin transport and its role in vascular differentiation is now being dissected at the molecular genetic level. With respect to leaf venation, the focus of this study, mutant phenotypes range from severely reduced venation in the *monopteros* (*mp*) auxin response factor mutant (Hardtke and Berleth, 1998), to

increased venation in the *pin-formed1* (*pin1*) auxin efflux carrier mutant (Galweiler *et al.*, 1998; Mattsson *et al.*, 1999). The roles of *MP* and *PIN1* in vein formation are also supported by their expression patterns and the subcellular distribution of the *PIN1* auxin carrier proteins (Scarpella *et al.*, 2006; Wenzel *et al.*, 2007). Their expressions undergo a gradual refinement before vein formation (Wenzel *et al.*, 2007), in line with the predictions of the canalization of auxin flow hypothesis (Sachs, 1981). Sachs' hypothesis also predicts that canalization drains auxin away from surrounding cells, thereby preventing their differentiation into vascular cells. Support for this conclusion comes from incremental pharmacological inhibition of auxin transport, which results in gradual vein overgrowth in the leaf lamina (Mattsson *et al.*, 1999; Sieburth, 1999). The distribution of vessel elements and the accumulated expression of the auxin response marker *DR5::GUS* in the leaf lamina suggest that the vascular overgrowth is due to auxin accumulation in the leaf lamina (Mattsson *et al.*, 2003).

Although leaf venation patterning shows remarkable plasticity in response to manipulation of auxin transport, other mechanisms are likely to play a role as well. In growing leaf primordia, the pattern and control of cell division activity in the procambial tissue, as well as in the complementary ground cell tissue, are likely to affect the final pattern and density of veins. Support for this notion comes from various studies. In *Flaveria bidentis*, relatively high vein density was partially explained by an early cessation of mesophyll cell divisions (McKown and Dengler, 2009). Ectopic expression of *AINTEGUMENTA*, which regulates cell division activity (Mizukami and Fischer, 2000), increases cell proliferation as well as the number of higher-order veins in *Arabidopsis* leaves (Kang *et al.*, 2007). In contrast, ectopic expression of *INHIBITOR OF CYCLIN DEPENDENT KINASE* decreases *Arabidopsis* leaf cell proliferation and the number of higher-order veins (Kang *et al.*, 2007). Leaf mesophyll cell differentiation may also terminate the progressive formation of higher-order veins in *Arabidopsis* (Scarpella *et al.*, 2004).

To date, there is no study assessing how cell division control, specifically in the procambial tissue or the complementary ground cell tissue, affects the patterning and density of leaf veins. This study addressed this question by inducing ectopic cell divisions, either in the procambial or in the ground tissue domain. To accomplish this, two complementary *GAL4*-green fluorescent protein (GFP) lines with vascular or ground tissue expression were used to drive *GAL4*-transactivated expression (Haseloff, 1999) of the *C4* protein from beet curly top virus (BCTV) specifically in these tissues. The *C4* protein induces ectopic cell divisions in plants (Latham *et al.*, 1997), coupled with induction of the cell-cycle-related genes *CYC*s, *CDK*s, *PCNA* (Park *et al.* 2010), and *RKP* (Lai *et al.*, 2009), resulting in activation of the host cell cycle to facilitate viral replication. BCTV *C4* protein bound to the AtSK η shaggy-related protein kinase in a yeast two-hybrid assay, indicating an interaction with the brassinosteroid pathway (Piroux *et al.*, 2007), although BCTV *C4* expression can affect multiple hormone pathways (Mills-Lujan and Deom, 2010). In *Arabidopsis*, cross-talk between the brassinosteroid and auxin pathways occurs via AtSK21 interaction with the auxin response factor ARF2 (Vert *et al.*, 2008), and shoot vascular patterning involves brassinosteroid signalling coupled to PAT (Ibañez *et al.*, 2009).

The present study examines the effect of BCTV *C4* targeted induction of cell divisions in developing ground or procambial

tissues and the correlated effects on auxin response patterns. Major alterations were observed in leaf growth and in vein patterning and density following *C4* expression in both procambial and ground tissues. Surprisingly, ectopic cell divisions in the ground cell tissue resulted in fragmentation of higher-order veins. This effect was strongly exacerbated by simultaneous inhibition of auxin transport, resulting in leaf blades almost entirely filled with vascular tissues and widespread expression of the auxin response marker *DR5::GUS*, suggesting that this synergistic phenotype was associated with increased auxin response or auxin content in both the ground and procambial tissues.

Materials and methods

GAL4-responsive UAS::C4 plasmid construction and plant transformation

The pJITC4 plasmid containing the *C4* coding region from BCTV (BCTV-*C4*) was kindly provided by J. Stanley (JIC Norwich) (Latham *et al.*, 1997). The *C4* coding region was used to replace the *KNAT3-YFP* sequence in a binary vector based on pBI121 (Jefferson *et al.*, 1987) that included five repeats of a modified *GAL4*-VP16 binding site upstream of the sequence for a *KNAT3-YFP* fusion protein, with the *C4* sequence bordered by a 5' *Bam*HI site and a 3' *Sac*I site (pBINKNAT3-YFP) (K. Siemering and J. Haseloff, unpublished; Haseloff, 1999). *C4* DNA was amplified by polymerase chain reaction using Vent DNA polymerase (New England Biolabs, Hitchin, UK) and the following primers: forward primer, 5'-GGCGGATCCAACAATGGGCAACCTCATCTCCAC; and reverse primer, 5'-GGCGAGCTCTTAACGCCTTGGCATATGAG. The forward primer introduced a *Bam*HI restriction site at the 5' end of the *C4* sequence and the reverse primer introduced a *Sac*I restriction site at the 3' end of the sequence. The modified *C4* DNA fragment was restricted with *Bam*HI and *Sac*I and ligated to *Bam*HI/*Sac*I digested DNA comprising the *GAL4*-VP16 binding sites from pBINKNAT3-YFP to form pBINC4 (designated UAS::C4). pBINC4 was electroporated into *Agrobacterium tumefaciens* strain LBA4044 and *Arabidopsis thaliana* ecotype C24 plants were subsequently transformed by the floral dip method (Clough and Bent, 1998).

Plant material and growth conditions

The *GAL4*-GFP enhancer trap lines Q0990 and J0571 were previously identified amongst a collection of *A. thaliana* lines generated in C24 ecotype (Haseloff, 1999). These lines are available from the Nottingham *Arabidopsis* Stock Centre (<http://nasc.nott.ac.uk>; Q0990, NASC ID N9217; J0571, NASC ID N9094). Three independent homozygous UAS::C4 lines (T4 generation), encoding *C4* under the control of *GAL4* upstream activator sequences (UASs), were generated. Each line was crossed to both homozygous *GAL4* enhancer trap lines, Q0990 and J0571, and detailed analyses performed on the F1 progeny (designated throughout as Q0990>>C4 or J0571>>C4). The three homozygous UAS::C4 lines conferred different strengths of phenotypic response when crossed to the *GAL4* lines, with line C4.1 < C4.2 < C4.3. F1 plants of J0571>>C4.2, Q0990>>C4.1, and Q0990>>C4.2 were also crossed with Col-0 plants containing the auxin-responsive promoter *DR5::GUS* construct (obtained from Tom Guilfoyle, University of Missouri, Columbia). Progeny containing the *GAL4* enhancer trap (Q0990 or J0571), UAS::C4, and *DR5::GUS* transgenes were examined for *DR5::GUS* response as described in Mattsson *et al.* (2003).

Seeds were either surface sterilized by agitation in 2% (v/v) commercial bleach (Domestos) for 15 min followed by several rinses in sterile water, or gas sterilized for 3 h in an enclosed chamber with 3% (v/v) HCl in commercial bleach. For selection of homozygous *GAL4-GFP* enhancer trap lines or UAS::C4 lines, seeds were plated in 9 cm Petri dishes on sterile *A. thaliana* salts (ATS) medium (Lincoln *et al.*, 1990) containing either 50 mg/l kanamycin or 50 mg/l hygromycin, respectively. For seedling growth analyses, plants were grown on either

ATS alone or with a final concentration of 10 μM *N*-naphthylphthalamic acid (NPA; TCI, Tokyo, Japan) to inhibit auxin transport. Seeds were stratified at 4 °C for at least 24 h and then grown in a controlled temperature growth chamber at 20 °C under constant fluorescent illumination ($\sim 100 \mu\text{mol m}^{-2} \text{s}^{-1}$). Plants used for analyses of developing embryos were grown in soil with 16 h daylight ($\sim 50\text{--}80 \mu\text{mol m}^{-2} \text{s}^{-1}$) at 20 °C.

Analyses of cellular architecture, GFP, and MP expression

GFP expression patterns of J0571 and Q0990 enhancer trap lines were analysed throughout development. Leaves or dissected embryos were fixed in 4% (w/v) paraformaldehyde in 0.025 M phosphate buffer for at least 2 h, rinsed in water, mounted in either glycerol/phosphate-buffered saline (1:1) or in 70% (v/v) aqueous glycerol and imaged for both GFP expression and background autofluorescence. Vascular patterning was examined in whole-mount 1–3-week-old seedlings or excised leaves, fixed and cleared as in Berleth and Jürgens (1993). *In situ* mRNA hybridizations of *MONOPTEROS* (*MP*) expression were performed on leaf primordia as in Wenzel *et al.* (2007) to determine the effects of *C4* misexpression on procambial strand formation.

Detailed analyses of the cellular architecture in mature embryos was examined in at least 50 F1 embryos of Q0990 or J0571 enhancer trap lines crossed to three independent UAS::*C4* lines. These were compared with at least ten of each of the mature control embryos: (i) wild type, *C24*; (ii) Q0990 or J0571 lines; (iii) UAS::*C4*; and (iv) F1 embryos of *C24*>>*C4* lines. Cellular architecture in Q0990>>*C4* and J0571>>*C4* embryos was compared throughout development with wild-type *C24* embryos. Embryos were cleared and cell walls stained with propidium iodide using a pseudo-Schiff reaction [described briefly in Haseloff (2003) and Moreno *et al.* (2006), and in more detail here]. Immature embryos were separated from developing siliques and the seed coat was either punctured (pre-heart stage) or removed (heart stage and older). Mature embryos were obtained by removing the seed coats of imbibed seeds. Embryos were fixed in methanol/acetic acid (5:1) overnight at 4 °C. The embryos were rinsed in distilled water, gently agitated in 1% (w/v) periodic acid for 30 min at room temperature, and then rinsed again in distilled water. Embryos were then immersed in aqueous Schiff reagent solution (0.1 M sodium metabisulphite, 1.5 μM HCl) containing 10% (v/v) of 1 mg ml^{-1} propidium iodide (Sigma, St. Louis, MO, USA) and gently agitated for 2 h at room temperature. The embryos were rinsed thoroughly in distilled water and placed in an aqueous 16 M chloral hydrate solution, transferred to a slide (excess chloral hydrate was removed), and mounted in Hoyer's solution (30 g gum arabic, 200 g chloral hydrate, 20 g glycerol, 50 ml water). Embryos were left to clear at least overnight to enable optical sectioning of the entire embryo.

Embryo cellular architecture and GFP images were captured with a Bio-Rad MRC 500 or a Zeiss 510 meta upright laser scanning microscope. For visualization of embryonic 3D cellular architecture, z-series were taken throughout the embryos at 0.1–0.4 μm intervals. A 543 nm excitation filter and 560 lp emission filter were used to visualize propidium iodide labelling of cell walls or autofluorescence after fixing in paraformaldehyde. GFP was visualized using 488 nm excitation and 505–550 nm emission filters. 3D reconstruction and visualization of the confocal z-series was performed with Amira version 2.1 (Visual Concepts, Germany) and ImageJ 1.34s (National Institute of Health, USA) software. Leaf vascular patterns and *MP* expression were imaged with a Nikon Eclipse E600 microscope and a Canon EOS D30 digital camera.

Results

Q0990 and J0571 lines drive complementary GFP expression in vascular and ground tissues during cotyledon and leaf development

Arabidopsis enhancer trap lines were previously generated through transformation with a T-DNA vector containing a modified GAL4-VP16 transcription activator gene and a GAL4-responsive mGFP5 gene under the control of GAL4 UASs (Haseloff, 1999).

Expression of the GAL4-VP16 gene is dependent on the proximity of *Arabidopsis* genomic enhancer elements, and the GAL4 protein then binds to UAS to drive tissue-specific expression of mGFP5 (Haseloff, 1999). The Q0990 and J0571 enhancer trap lines drive GFP expression in procambial or ground tissue respectively. Ectopic cell divisions were induced through GAL4 driven expression of the BCTV C4 protein in the procambial and ground tissues following genetic crosses between the GAL4 lines Q0990 and J0571, respectively, and the GAL4-responsive UAS C4 lines.

In the Q0990 line, GFP expression was already evident in the procambium and ground tissues of the root and hypocotyl regions in late globular-stage embryos (data not shown). GFP expression became restricted to the vascular and pericycle cells by the early heart stage, and thereafter almost exclusively in the vascular tissues (Fig. 1B) including seedling leaf venation (Fig. 2A–D). *A. thaliana* leaves have a hierarchical reticulate venation patterning, with primary and secondary lower-order veins and tertiary and quaternary higher-order veins (Mattsson *et al.*, 1999; Kang and Dengler, 2002). In cotyledons and leaves, GFP expression was observed in the developing primary vein, in secondary veins forming loops on either side of the primary vein, and also in higher-order veins (Figs. 1B, 2A–C). Hence GFP expression in Q0990 occurred in preprocambial and procambial cells of embryos, and, as also determined by Sawchuk *et al.* (2007), in developing procambial cells of leaves (e.g. Figs. 1B, 2A–D). Q0990 GFP expression in leaf was eventually reduced or lost in a basipetal direction as leaf veins matured (e.g. Fig. 2D).

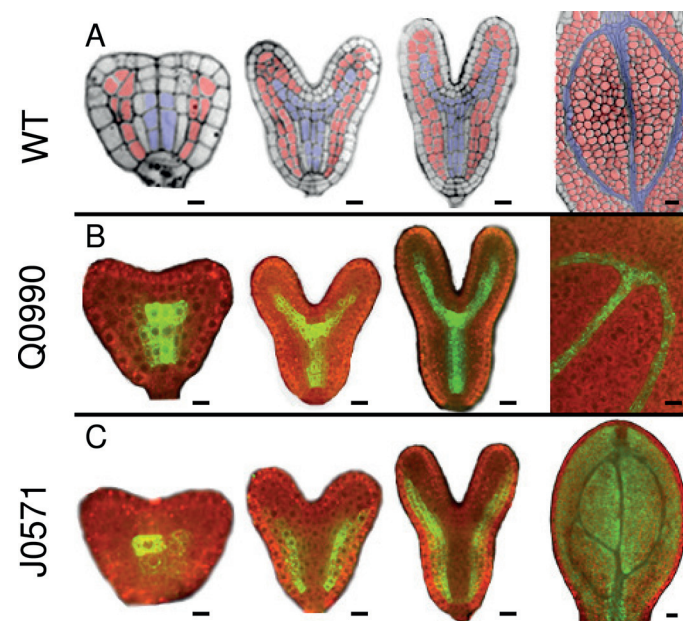


Fig. 1. GFP expression in vascular (Q0990) and ground (J0571) tissues during embryo development. Confocal images show vascular tissues (blue) and ground tissues (red) in wild-type embryos (A) or GFP expression in, Q0990 (B) and J0571 (C) embryos at different developmental stages (left to right: triangular/early heart, late heart, torpedo, mature embryonic cotyledon). All images are median longitudinal sections except the early heart stage of J0571, which is a tangential longitudinal section taken through the ground tissue showing GFP expression in these cells. Bars, 10 μm .

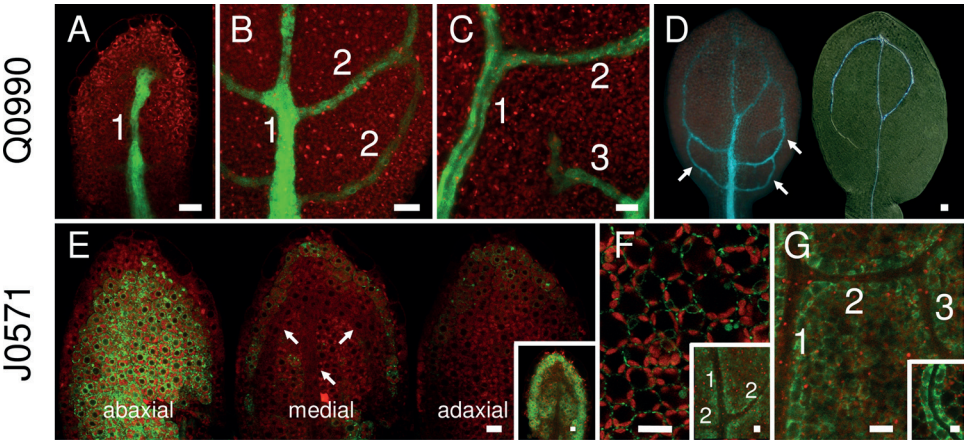


Fig. 2. GFP expression in vascular (Q0990) or ground (J0571) tissues during leaf development. Images show first rosette leaf primordia at 2 (A), 3 (E), 4 (B, F inset), 5 (C, D, G), 6 (F), and 7 (G inset) days after germination. (A–C) GFP expression in Q0990 leaf primordia was observed progressively in the primary midvein (1), secondary veins (2), and tertiary veins (3). (D) In Q0990, GFP fluorescence (left) and cleared darkfield image of the same leaf showing differentiated xylem (right) indicate that GFP expression was also present in undifferentiated procambial cells (arrows). (E–G) In J0571, GFP expression was initially mostly in abaxial tissue (arrows in medial section show procambial cells) (E) and progressively included more adaxial ground tissues (E inset, F), increasingly delimiting venation (F inset, G), and eventually becoming more prominent in cells directly surrounding the vascular tissue (G inset). Bars, 10 μm (A–C, E–G), 20 μm (D).

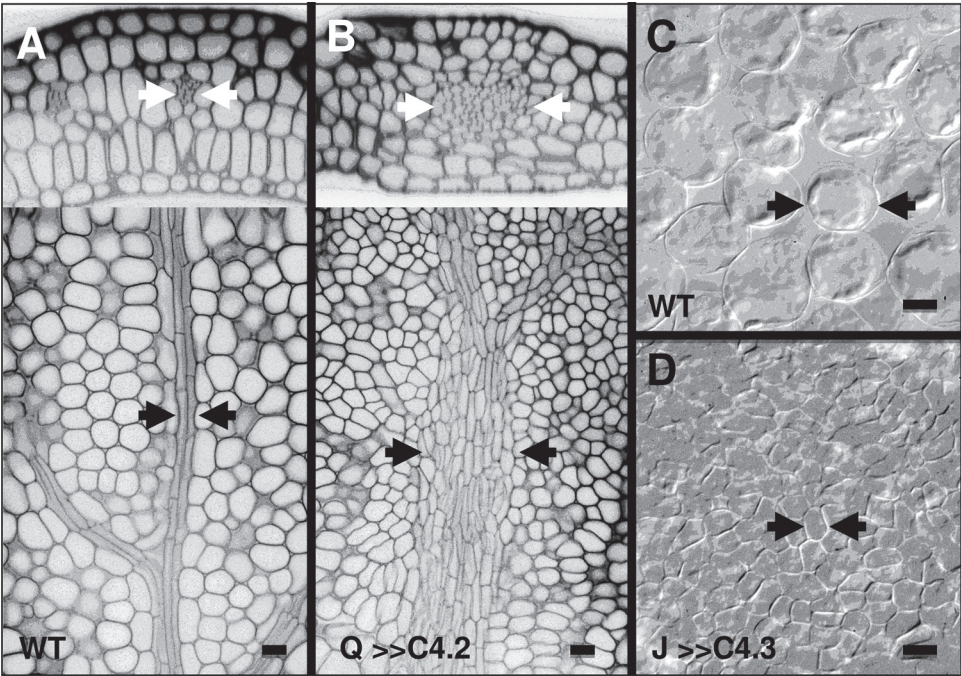


Fig. 3. Effects of C4 transactivation on cell number. Confocal (A, B) or Nomarski (C, D) images show that C4 increased the number of vascular cells in the embryonic cotyledon midvein of Q0990>>C4.2 (B) compared with wild type (A), as seen in transverse (top) and longitudinal (bottom) sections of the same cotyledons. Leaves of 2-week-old J0571>>C4.1 plants had more numerous, smaller palisade cells with smaller or no intercellular spaces (D) compared with wild type (C). Arrows delimit vascular bundles (A, B) or a typical mesophyll cell (C, D). Q, Q0990; J, J0571. Bars, 10 μm .

In contrast to Q0990, GFP expression in J0571 was restricted predominantly to ground tissues in developing embryos and seedlings. GFP was observed in the developing root and hypocotyl regions from early heart-stage embryos onwards (Fig. 1C). In late heart and torpedo-stage embryos, GFP was also expressed in

the abaxial ground tissues of the cotyledons, eventually including medial and adaxial ground cells (data not shown) and clearly outlining vascular tissues in more mature cotyledons (Fig. 1C). In emerging and young leaf primordia (e.g. Fig. 2E, 2–3 days after germination, DAG), GFP was predominantly observed in

the abaxial ground tissues and in the ground cell layer underlying the epidermis, although some leaf primordia also had GFP expression in more medial and adaxial tissues (Fig. 2E inset). In more mature leaf primordia (e.g. Fig. 2F inset, G, 4–6 DAG), GFP expression in ground cells eventually delimited the developing primary, secondary, and higher-order veins, and included more adaxial ground tissues. In older J0571 leaves (e.g. Fig. 2G inset, 7 DAG), GFP expression was most abundant in ground cells directly adjacent to the vascular bundles.

In summary, Q0990 and J0571 lines showed procambial and ground tissue GFP expression, respectively, throughout embryonic cotyledon and postembryonic leaf development, and were therefore suitable to drive GAL4-activated C4 expression in these tissues. GFP expression in all leaf procambial cells (Q0990) or the potential procambial domain of tertiary veins (J0571) occurred later than expression of two of the earliest known preprocambial markers (*MONOPTEROS* mRNA and PINFORMED-1 protein; Wenzel *et al.*, 2007), but approximately concurrently with procambial expression of the auxin response marker *DR5::GUS* (Mattsson *et al.*, 2003). Thus, the effect of C4 transactivation would only start at the developmental stages corresponding to Q0990 and J0571 GFP expression patterns.

C4-induced ectopic cell divisions

To confirm the assumption that targeted C4 expression would induce ectopic cell divisions, the effects were analysed at the anatomical level. For Q0990>>C4 lines, data were taken from embryonic cotyledons because only one of the three analysed lines, Q0990>>C4.1, consistently produced leaves. Data was also taken for mature leaves of J0571>>C4 lines because the leaves had significant vascular patterning defects. Compared to the controls, C4 expression in developing procambial cells of Q0990>>C4 resulted in ectopic vascular cell production (Fig. 3B compared with A), without concurrent increases in embryonic cotyledon size (Fig. 4A) or mesophyll cell number (i) within a cross-sectional area (Fig. 4B) or (ii) along the length or width of the cotyledon (data not shown). Compared to controls, expression of C4 in developing ground tissues of J0571>>C4 resulted in a larger number of smaller leaf mesophyll cells with small or no intercellular spaces between them (Fig. 3D compared with C, Fig. 4C). J0571>>C4 leaves of 2-week-old plants were slightly larger than controls (Fig. 4A) and had similar leaf vein width and vascular cell widths compared to controls (data not shown), thus showing no apparent ectopic vascular production. Hence, C4 expression did result in ectopic procambial and ground cell divisions in Q0990>>C4 or J0571>>C4 lines, respectively. The extent of induced divisions varied with the different C4 lines, with C4.1 < C4.2 < C4.3 (Fig. 4B, 4C).

Expression of C4 in vascular and ground tissues affects seedling morphology

Expression of C4 in vascular (Q0990>>C4) or ground (J0571>>C4) tissues had pleiotropic effects on seedling morphology, with the severity of phenotypic effects varying between different C4 lines (C4.1 < C4.2 < C4.3), correlating with the extent of induced divisions. Q0990>>C4.1 seedlings had weaker phenotypes and were able to produce leaves, which often bent adaxially and/or had

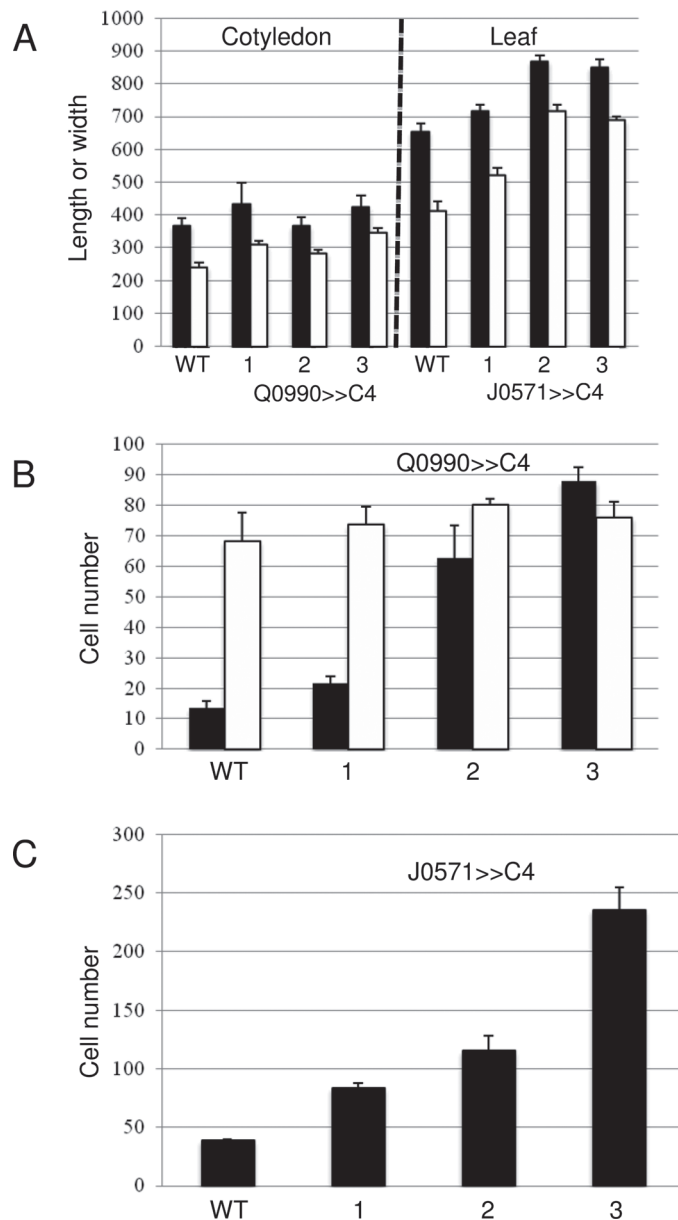


Fig. 4. Effects of C4 transactivation on Q0990>>C4 embryonic cotyledons and 2-week-old J0571>>C4 third leaf sizes (A) and cell numbers (B, C). (A) Compared to WT, Q0990>>C4 (left) had similar sized embryonic cotyledons and J0571>>C4 (right) had slightly larger third leaves in 2-week-old plants; length (black) or width (white) of cotyledons (μm) or leaves (mm x 10⁻²). (B, C) Compared to WT, Q0990>>C4 embryonic cotyledons had more midvein vascular cells (black) without concurrent increases in mesophyll cell number (white) (B) and J0571>>C4 leaves had more mesophyll cells (C); cell number data are for ~63,000 μm² of field of view for cross-sections (B) and longitudinal sections (C). Values are mean ± SE for ≥3 leaves/cotyledons. 1, C4.1; 2, C4.2; 3, C4.3.

twisted petioles that altered leaf positioning (Fig. 5B). For the two strongest Q0990>>C4 lines, leaf primordia were absent or incompletely formed and hypocotyls had tumour-like vascular protrusions (Fig. 5C). Inflorescence stems were stunted (data not shown). All J0571>>C4 seedlings had twisted, curled, or rolled leaves

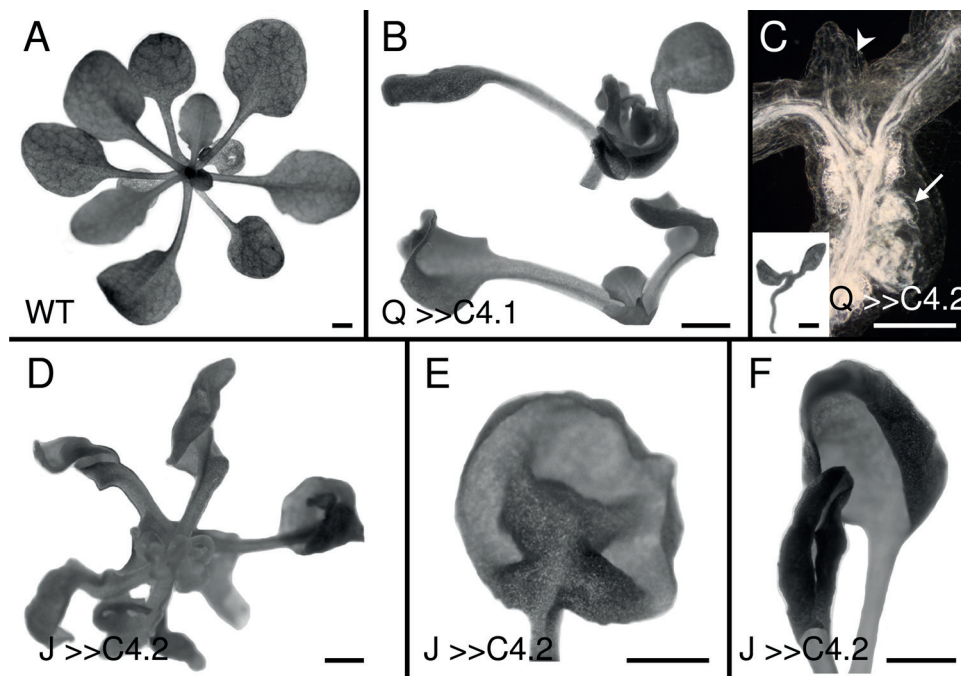


Fig. 5. Effects of C4 transactivation on 2–3-week-old seedling morphology of wild type (A), Q0990>>C4.1 (B), Q0990>>C4.2 (C), or J0571>>C4.2 (D–F). (B) Q0990>>C4.1 plants had curled leaves and petioles. (C) Q0990>>C4.2 seedlings were stunted and had limited leaf growth (arrowhead and inset) and exhibited tumour-like vascular overgrowth in the hypocotyl (arrow). (D–F) J0571>>C4.2 seedling leaves were twisted (D), buckled (E), or rolled (F). Q, Q0990; J, J0571. Bars, 1 mm (A, B, C inset, D–F), 0.5 mm (C darkfield image).

(Fig. 5D–F) and stunted, twisted inflorescence stems (data not shown). Although J0571>>C4 leaves were initially slightly larger than controls (see above), the continuous induction of cell divisions was detrimental and resulted in an overall stunted mature phenotype.

C4 expression in procambial cells results in disrupted and ectopic veins

Dark-field microscopy of cleared specimens was used to determine whether ectopic procambial divisions affected cotyledon and leaf venation patterning. In each of the Q0990>>C4 lines, more than 80% of seedlings ($n \geq 30$) had defective cotyledon venation (Fig. 6C–G) compared with wild type (Fig. 6A, 6B). In the two weaker lines (Q0990>>C4.1 and Q0990>>C4.2), cotyledons typically had short, discontinuous veins adjacent to or extending from primary and secondary veins, and the secondary vein loops were often discontinuous (e.g. Fig. 6C–E). In more extreme cases, embryonic and seedling cotyledons had multiple ectopic veins in the midvein region and short secondary-like veins only partially extending towards the margin (Fig. 6E–G). In Q0990>>C4.2 and Q0990>>C4.3, leaf growth was absent or severely inhibited, and leaves often had only partial secondary vein formation (Fig. 7D). Q0990>>C4.1 seedlings produced relatively normal sized leaves, but more than 98% of leaves ($n \geq 50$) had discontinuous secondary or tertiary veins (Fig. 7B, 7C), and patterning of secondary loops was often aberrant (Fig. 7B compared with A). To determine if aberrant secondary vein formation was correlated with abnormal cell division orientation patterns, cell division orientation was examined in Q0990>>C4 plants. Unlike control plants, Q0990>>C4 secondary procambial veins often had cells with division planes perpendicular or close to perpendicular to the axis

of the vein (Fig. 8B compared with A) and partial veins often terminated adjacent to an apparently undivided cell (data not shown). For example, Q0990>>C4.2 embryonic cotyledons ($n \geq 10$) had 1.8 ± 0.2 partial secondary veins, whereas the controls only formed looped secondary veins. Hence, ectopic expression of C4 in the procambial domain induced ectopic vascular strands, but in more severe cases it disrupted overall patterning often leading to a reduction of secondary and tertiary venation.

Auxin transport inhibition restores vein continuity in Q0990>>C4.1 leaves

The ectopic vessels and vessel elements found in cotyledons of Q0990>>C4.2 plants were similar to those caused by auxin transport inhibition (ATI) through exposure to NPA (Fig. 6H, 6I, Fig. 7E). These effects of NPA treatment have previously been described (Mattsson *et al.*, 1999; Sieburth, 1999). If induced ectopic expression in the procambial domain and auxin transport inhibition contributed independently to the formation of ectopic vessels and vessel elements, a combination of the two treatments should result in a synergistic, exaggerated phenotype. To test this hypothesis, the effect of NPA on vein development was examined in Q0990>>C4.1 seedlings, which are able to form true leaves. Leaves of Q0990>>C4.1 seedlings grown on medium supplemented with NPA (Fig. 7F, 7G) displayed vein overgrowth similar to the effects of NPA alone (Fig. 7E). NPA treatment reduced the frequency of Q0990>>C4.1 leaves with vein continuity defects from ~95% (control, $n \geq 50$) to ~5% (NPA-treated; $n \geq 30$) (Fig. 7F). The remaining 5% showed primarily stubby vein protrusions from the midvein (e.g. Fig. 7G arrow), a defect that also occurred in ~20% of wild-type plants exposed to NPA (data not

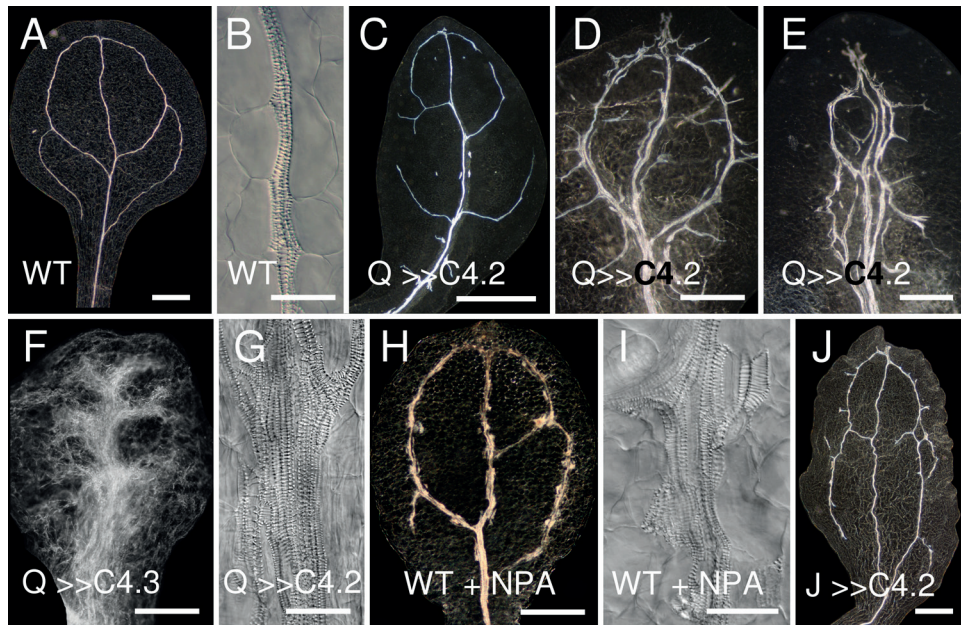


Fig. 6. Effects of *C4* transactivation on cotyledon venation. Images show cleared cotyledons of ~2-week-old plants. Darkfield (A, C–F, H, J) or Nomarski (B, G, I) images of wild-type (A, B, H, I), Q0990>>C4.2 (C–E, G), Q0990>>C4.3 (F), and J0571>>C4.2 (J) cotyledons grown on control ATS media (A–G, J) or ATS media supplemented with 10 μ M NPA (H, I). *C4* induced ectopic vascular strand formation similar to the effects of *N*-naphthylphthalamic acid. Q, Q0990; J, J0571. Bars, 500 μ m (A, C–F, H, J), 50 μ m (B, G, I).

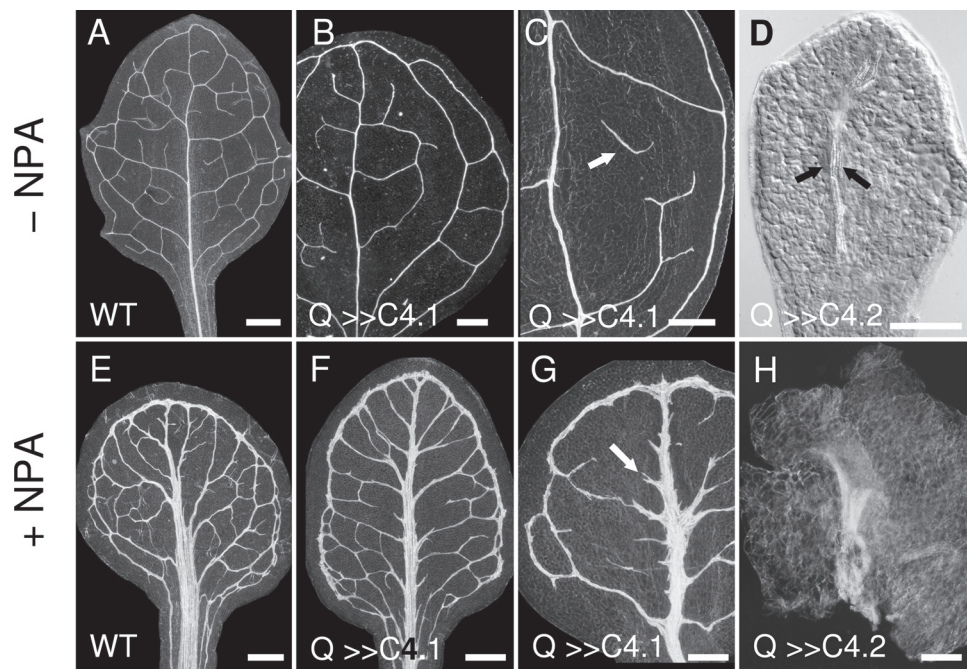


Fig. 7. Q0990>>C4 leaf venation patterning. Darkfield (A–C, E–H) or Nomarski (D) images of 2-week-old wild-type (A, E), Q0990>>C4.1 (B, C, F, G), or Q0990>>C4.2 (D, H) leaves grown on control media (A–D) or media supplemented with 10 μ M *N*-naphthylphthalamic acid (NPA; E–H). Q0990>>C4.1 leaves had distorted secondary venation patterning and discontinuous secondary and higher-order veins (B, arrow in C), while Q0990>>C4.2 leaves often lacked secondary vein formation (D; arrows delimit midvein). NPA treatment induced vascular overgrowth in wild-type (E) and Q0990>>C4 (F–H) leaves, although vein discontinuities were still evident in some Q0990>>C4.1 leaves (e.g. arrow in G) and in Q0990>>C4.2 (H, veins barely extending from midvein region). Q, Q0990. Bars, 500 μ m (A–C, E–G), 100 μ m (D, H).

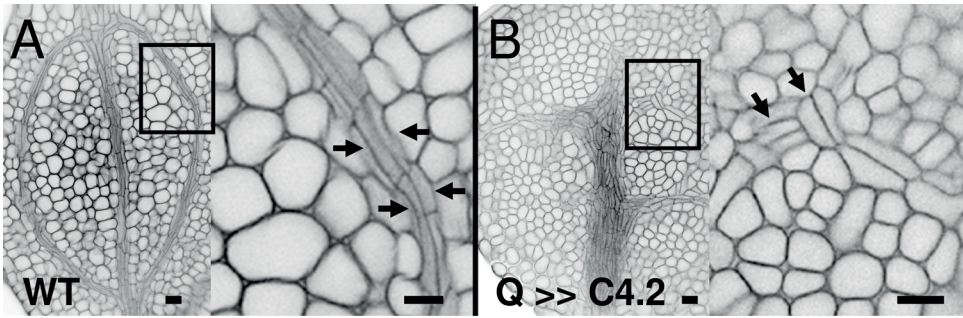


Fig. 8. Q0990>>C4 procambial cell orientation. Confocal images of embryonic cotyledons of wild type (A) and Q0990>>C4.2 (B), showing higher magnification of boxed regions to the right. Compared with the highly aligned procambial cells of wild-type secondary veins (arrows in A), Q0990>>C4.2 procambial cells were often not in parallel orientations (arrows in B). Q, Q0990. Bars, 10 μ m.

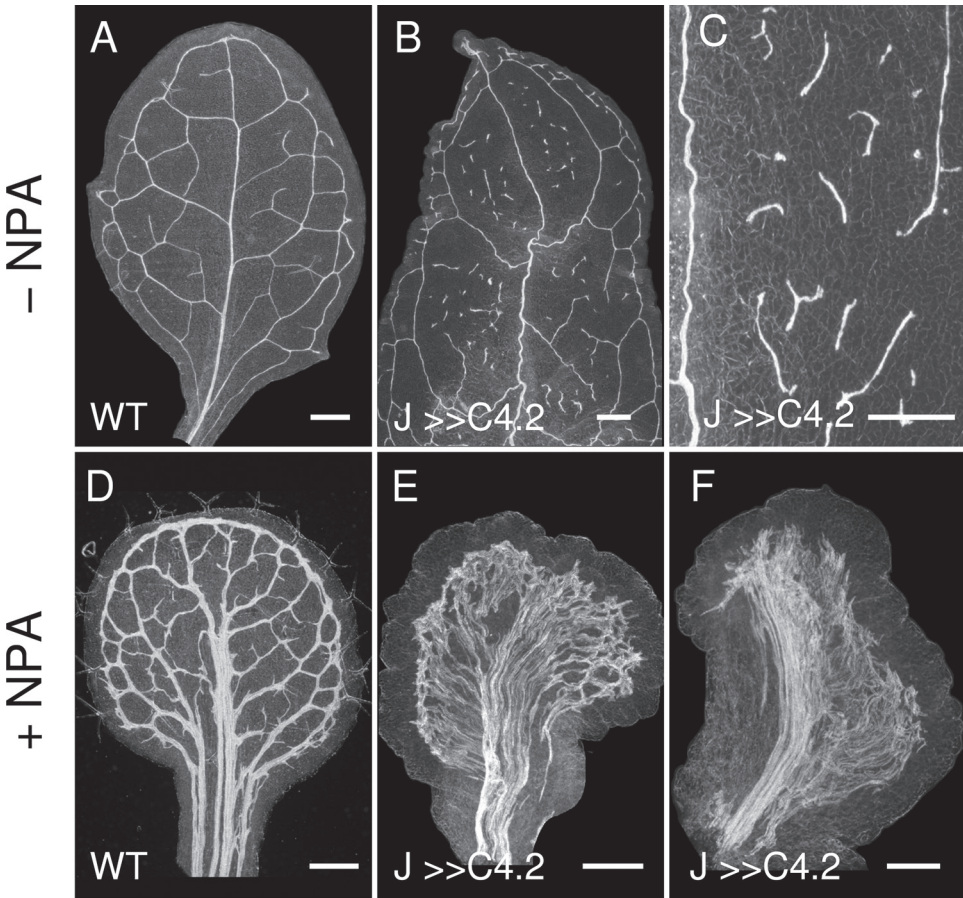


Fig. 9. J0571>>C4 leaf venation patterning. Darkfield images of 2-week-old wild-type (A, D) and J0571>>C4.2 (B, C, E, F) leaves grown on control ATS media (A–C) or ATS media supplemented with 10 μ M *N*-naphthylphthalamic acid (NPA; D–F). J0571>>C4.2 leaves had discontinuous tertiary and higher-order veins (B, C), and with NPA treatment had more extensive vascular overgrowth throughout most (E) or predominantly one side (F) of the lamina compared with wild type (D). J, J0571. Bars, 500 μ m.

shown). NPA treatment of Q0990>>C4.2 seedlings, which produce small and highly defective leaves, did induce some vascular overgrowth in the midvein region, but did not restore growth of secondary veins (Fig. 7H). In summary, the combination of ectopic procambial cell divisions and ATI did not result in an exaggerated phenotype. Instead, ATI either had little or no effect (the strong Q0990>>C4.2 line) or mostly masked the defects (the weaker Q0990>>C4.1 line), including restoring continuity of secondary veins. From a genetic perspective, this effect is analogous

to an epistatic relationship, which would suggest that the two defects affect the same pathway (see also Discussion).

Ectopic cell divisions in ground tissue disrupt higher-order venation

Although leaf procambial cells differentiate from ground meristem cells, the likely response of leaf venation to ectopic ground cell divisions is not obvious and would depend on the timing

of cell division induction. If additional ground cells contribute only to mesophyll cells, one would expect larger areoles (areas between major vein loops). If, on the other hand, the ground cells contribute additional cells for vein formation, one might expect higher vein complexity. To test this, C4 transactivation in J0571>>C4 was used to induce ectopic cell divisions in ground tissue. In each of the J0571>>C4 lines, most seedlings ($n \geq 30$) had normal cotyledon vascular patterning, but ~30% of cotyledons displayed short ectopic vascular strands and/or vascular strand discontinuities (Fig. 6J). All J0571>>C4 lines had continuous, albeit distorted, primary and secondary veins in leaves, but severely disrupted higher-order venation, particularly in J0571>>C4.2 (Fig. 9B, 9C) and J0571>>C4.3 (data not shown). In summary, induced ground cell divisions disrupted minor but not major veins. In J0571, GFP expression was observed in medial tissues (from which potential procambial cells would be recruited) predominantly from 4 DAG onwards, after the formation of the primary and secondary veins. Hence, the effect of ectopic ground cell divisions on primary and secondary vein formation could not be determined using the J0571 line. Higher-order veins are formed in slightly older leaf primordia (Wenzel *et al.*, 2007), which would coincide with the presumed C4 transactivation in GFP-expressing ground cells of J0571>>C4, thus resulting in predominant disruption of higher-order venation.

Ectopic ground cell divisions and auxin transport inhibition result in massive vein overgrowth

To determine the combined effects of ectopic ground cell divisions and ATI, J0571>>C4 seeds were germinated and grown in the presence of NPA. Seedlings of J0571>>C4.2 treated with NPA developed leaves with extreme vascular overgrowth (Fig. 9E; $n \geq 30$). The vascular strands of NPA-treated leaves extended from near the leaf margin towards the primary vein region and covered almost the entire laminar area, excluding the margins (Fig. 9E). In ~5% of leaves ($n > 30$), vascular strands were absent on either one or both sides of the central lamina region, correlated with inhibited lateral lamina growth (Fig. 9F). In summary, the combination of ectopic ground cell divisions and ATI resulted in a novel phenotype, comprising a massive vascular overgrowth in leaves (Fig. 9E, 9F), not present in leaves from either of the two single treatments (Fig. 9B–D). From a genetic perspective, this would be analogous to a synergistic effect, indicating that both treatments contributed to the formation of leaf veins independently (see also Discussion).

Vein disruptions were due to defects in vascular differentiation

To understand whether the vein discontinuities observed in Q0990>>C4 and J0571>>C4 leaves were caused during procambial strand formation or subsequent vein differentiation, *MONOPTEROS* (*MP*) expression was examined during procambial strand formation in Q0990>>C4.1 and J0571>>C4.2 plants. The expression of *MONOPTEROS*, an auxin response factor, is one of the earliest known markers of leaf procambial cell fate (Wenzel *et al.*, 2007). In leaves of wild-type C24 seedlings grown in control media, *MP* was expressed in all developing procambial

strands (Fig. 10A). *MP* expression was similar to that of wild type in leaves of most Q0990>>C4.1 and all J0571>>C4.2 seedlings, indicating formation of continuous procambial strands in these plants (Fig. 10B). Thus, discontinuous veins were due to defects in subsequent vascular differentiation rather than formation of procambial strands, resulting in aberrant differentiation along vein strands (Fig. 10D). *MP* expression was also examined in more severe Q0990>>C4.2 phenotypes with aberrant secondary vein patterning to determine if procambial strand formation was defective. Q0990>>C4.2 leaves had *MP* expression in the primary midvein and in short secondary-like strands partially extending from the primary vein (Fig. 10C), indicating that continuous secondary procambial strand loops were not formed. Hence, while vein discontinuities were caused by defects in differentiation, more severely perturbed vascular patterning defects

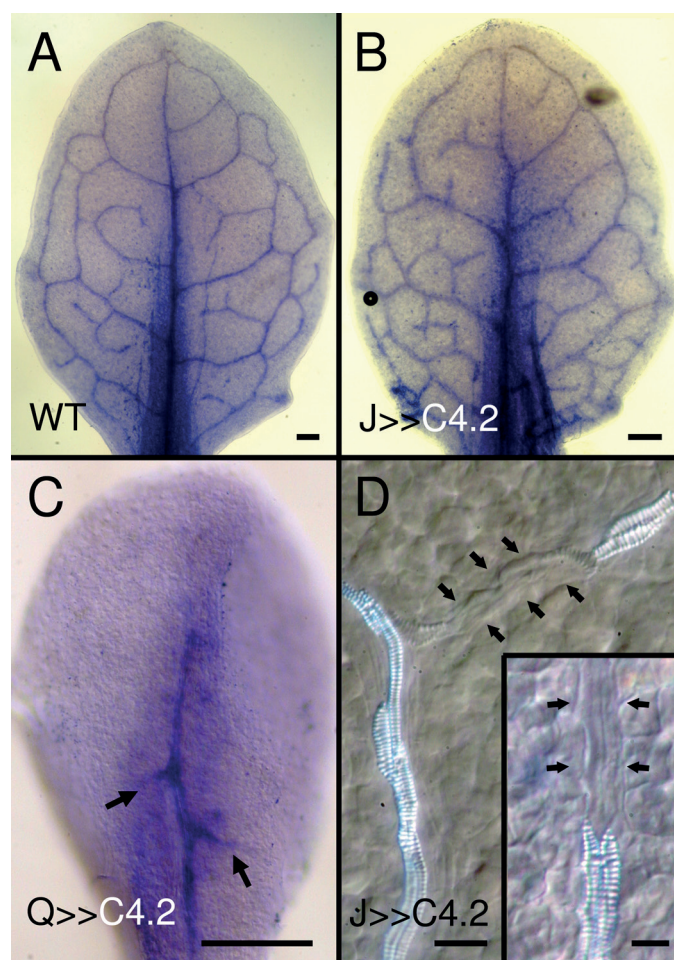


Fig. 10. *MP* expression in leaves. Whole-mount *in situ* hybridizations of *MP* mRNA transcript in rosette leaves of wild type (A), J0571>>C4.2 (B, D), or Q0990>>C4.2 (C) grown for ~1 week on control media. In wild type and J0571>>C4.2, *MP* was expressed in procambial cells of all vein types (A, B). *MP* was only expressed in the primary vein and in short abutting secondary veins in Q0990>>C4.2 (C, arrows). J0571>>C4.2 leaves had normal *MP* expression (B), but had punctate differentiation along vascular strands (arrows, D and inset). Q, Q0990; J, J0571. Bars 100 μ m (A–C), 10 μ m (D).

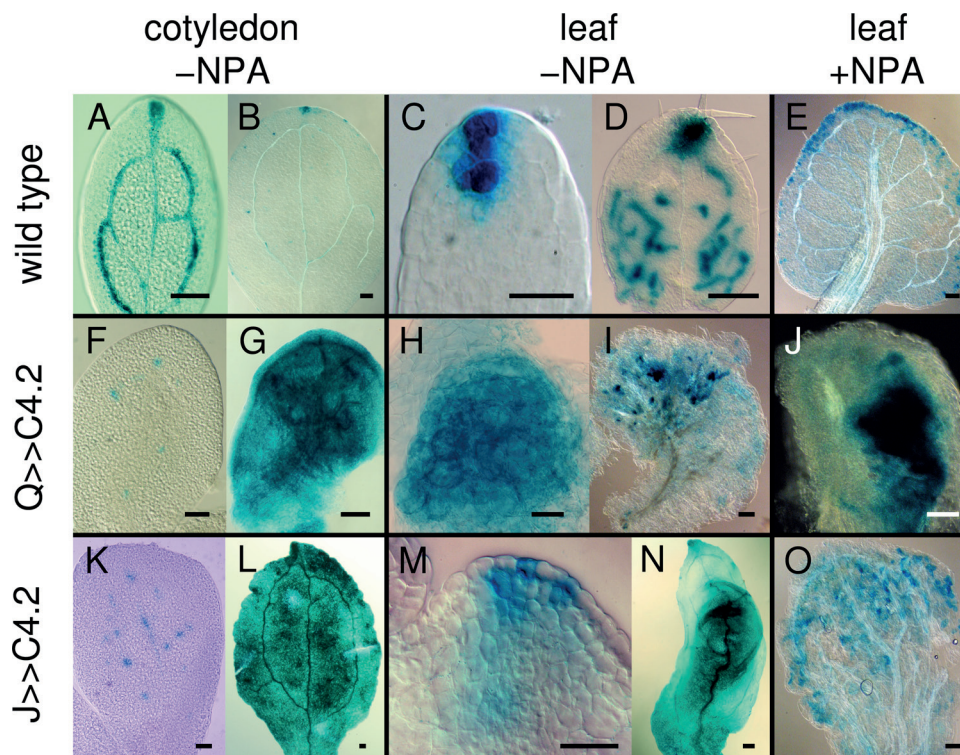


Fig. 11. Effects of *C4* transactivation on *DR5::GUS* expression in leaves. *DR5::GUS* expression in wild-type (A–E), Q0990>>C4.2 (F–J) or J0571>>C4.2 (K–O) mature embryonic cotyledons (A, F, K), 7 days after germination cotyledons (B, G, L), 2–3 days after germination leaf primordia (C, H, M), or 6–8 days after germination leaves (D, E, I, J, N, O) grown in control media (–NPA; A–D, F–I, K–N) or media supplemented with 10 μ M *N*-naphthylphthalamic acid (+NPA; E, J, O). *C4* transactivation resulted in ectopic *DR5::GUS* expression. Q, Q0990; J, J0571. Bars, 100 μ m (B, D, E, G, I, L, N, O), 20 μ m (A, C, F, H, K, M).

were associated with defective procambial strand formation.

Ectopic cell divisions disrupt formation of discrete auxin response maxima

Cotyledon and leaf primordia display auxin response maxima in procambial cells as well as apical and lateral auxin maxima (Aloni *et al.*, 2003; Mattsson *et al.*, 2003). To assess the effect of ectopic cell division activity in the procambial and ground tissues on the distribution of auxin response maxima, expression of the auxin-responsive *DR5* promoter fused to GUS was examined. Ectopic divisions in both Q0990>>C4.2 and J0571>>C4.2 disrupted discrete auxin response maxima formation in procambial cells and in the margin convergent sites where auxin presumably would flow from the epidermis towards the developing procambial cells (Scarpella *et al.*, 2006; Wenzel *et al.*, 2007). In wild type, mature embryonic cotyledons had *DR5* expression in apical auxin maxima and procambial cells (Fig. 11A), but by 7 DAG, *DR5* was predominantly expressed in the apical auxin response maxima (Fig. 11B). In 1–2 DAG wild-type leaf 1 primordia, *DR5* was expressed in a few apical cells and also in developing procambial cells up to 7 DAG (Fig. 11C, 11D), after which it became restricted to apical and lateral marginal auxin maxima in the hydathodes (data not shown). Both Q0990>>C4.2 (with severe vascular defects) and J0571>>C4.2 mature cotyledons had sporadic, punctate *DR5* expression (Fig. 11F, 11K), with ubiquitous *DR5*

expression throughout the cotyledon in germinating seedlings up to ~8 DAG (Fig. 11G, 11L), after which it decreased again (data not shown). Both Q0990>>C4.2 (with severe vascular defects; Fig. 11H, 11I) and J0571>>C4.2 (Fig. 11M, 11N) had broad *DR5* expression in early leaf primordia and more diffuse expression throughout the lamina of older leaves. This effect was not entirely due to disruption of auxin transport, because NPA-grown leaves had *DR5::GUS* expression near the leaf margins in wild-type leaves (Fig. 11E), but more sporadically throughout the lamina in Q0990>>C4.2 (Fig. 11J) and J0571>>C4.2 (Fig. 11O) seedlings. Taken together, ectopic cell divisions in procambial or ground cells disrupted formation of auxin response maxima and frequently resulted in *DR5::GUS* expression throughout most of the leaf lamina.

Discussion

The formation of discrete veins with continuous differentiation along strands requires precise control of cell division

Although the venation patterns of dicotyledonous leaves are regular enough to be used as phylogenetic traits (Hickey, 1979; Ellis *et al.*, 2006), leaf venation patterns are not identical, even from leaves of the same individual. This phenotypic plasticity has been attributed to stochastic events during pattern formation, possibly due to a positive feedback mechanism referred to as

canalization of auxin flow (Sachs, 1991), with vein patterning and densities dramatically changing upon pharmacological ATI (Mattsson *et al.*, 1999; Sieburth, 1999). Here, the extent to which the plasticity in venation patterning and discrete vein formation respond to ectopic cell divisions was assessed by partly uncoupling cell division activity from its normal regulation, using BCTV-C4 induction of ectopic cell divisions in targeted vascular or ground tissue domains.

Ectopic divisions within the procambial domain had varying effects on cotyledon and leaf vascular patterning. Slight perturbations in procambial cell divisions had little effect on overall vascular patterning but sometimes prevented continuous differentiation along veins, possibly by disturbing regular cell division activity and/or improper alignment of procambial cells, which may have affected auxin transport (see below). More severe perturbations resulted in ectopic veins and vessels, demonstrating the need for controlled procambial divisions in the formation of discrete veins with specific cellular architectures. High rates of ectopic procambial divisions resulted in misaligned procambial cells and discontinuous procambial cell files, impeding secondary and higher-order vein formation and even leaf formation/growth, indicating that both vein patterning and leaf formation/growth collapsed without adequate control of cell division activity within the procambial domain.

Intuitively, ectopic ground cell divisions might be expected to result in enlarged leaves and possibly also more veins, because veins originate from the medial ground tissues. Although ectopic ground cell divisions resulted in slightly larger and non-planar leaves, no obvious increase in venation was observed. Instead, extensive fragmentation of higher-order veins occurred even though the procambial veins were continuous, suggesting that vein differentiation was more sensitive to the unscheduled cell divisions. However, J0571 GFP expression in the medial ground tissue predominantly occurred after procambial formation, suggesting that fewer unscheduled cell divisions may have occurred during procambial vein patterning rather than during vein differentiation. This discontinuous vein phenotype is similar to that of the vascular continuity mutant *vascular network3* (*van3*; Scarpella *et al.*, 2006), which is defective in the subcellular localization of plasma membrane proteins, including PIN-FORMED auxin efflux carriers (Naramoto *et al.*, 2010). Hence, it is possible that ectopic ground cell divisions physically displaced procambial cells in tertiary veins and thereby possibly also inducing unscheduled procambial divisions, disrupting the auxin transport required for differentiation of continuous veins. In addition to auxin, vascular differentiation involves other hormones and genetic regulators (reviewed in Caño-Delgado *et al.*, 2010; Ohashi-Ito and Fukuda, 2010) whose activity or distribution may have also been disrupted as a result of unscheduled ground cell divisions.

Ectopic cell division activity in the procambial domain may also disrupt auxin transport

Ectopic procambial cell divisions resulted in vascular defects reminiscent of the effects of PAT inhibition, particularly in the midvein region of severe Q0990>>C4 cotyledon phenotypes, with wider veins and defective connections between vessel

elements. While ATI is likely to result in the selection of wider ground cell domains for procambial differentiation (Mattsson *et al.*, 1999; Sieburth, 1999; Mattsson *et al.*, 2003), ectopic procambial cell divisions may have a similar net effect by triggering additional divisions in existing procambial cells. The imperfect interconnection of vessel elements observed here could be due to disturbances in auxin transport, caused by new unpolarized cells disrupting the intercellular auxin transport that is believed to enhance alignment and interconnection of procambial cells. However, it should be noted that while both ground and procambial ectopic cell divisions affected vein continuity, with similarities to previously described auxin-related phenotypes, the leaf phenotypic defects from ectopic procambial divisions can be much more profound than those resulting from ectopic ground cell divisions (see below).

The C4 protein itself and/or C4-induced disrupted vascular patterning observed in this study are likely to have affected other hormonal pathways. The BCTV C4 protein interacts with AtSK η shaggy-related protein kinase in the brassinosteroid signalling pathway (Piroux *et al.*, 2007), although constitutive expression of BCTV-C4 can affect multiple hormonal pathways in *Arabidopsis* (Mills-Lujan and Deom, 2010). In *Arabidopsis*, cross-talk between the brassinosteroid and auxin pathways occurs via AtSK21 action on the auxin response factor ARF2 (Vert *et al.*, 2008), and determination of shoot vascular bundle number involves brassinosteroid signalling coupled to PAT (Ibañez *et al.*, 2009). The brassinosteroid pathway may have also influenced vascular overproduction in Q0990>>C4, as well as factors such as *ACAULIS5* (Clay and Nelson, 2005), *TORNADO1* (*TRN1*), and *TRN2* (Cnops *et al.*, 2006) that can affect leaf vascular cell divisions and auxin distribution.

Ectopic procambial divisions in leaves of the weaker transactivation line Q0990>>C4.1 also resulted in disruptions in leaf vein continuity. When ectopic cell divisions and ATI were combined, analogous to the construction of a double mutant line, vein continuity was restored and most leaves showed the stronger vascular overproduction phenotype typical of ATI alone. Hence, ATI masked or, if expressed in analogous genetic terms, acted epistatically to ectopic cell divisions, suggesting that both perturbations acted at least broadly on the same pathway, with ATI having the dominant influence on the outcome. This is similar to the situation for FORKED1, which influences PIN1 localization in an auxin dependent manner (Hou *et al.*, 2010). *forked1* vein continuity is also restored during ATI, presumably by restoring sufficient auxin concentrations for continuous differentiation along vascular strands (Steynen and Schultz, 2003; Hou *et al.*, 2010).

Stronger C4 effects on procambial activity resulted in reduced lamina expansion, coupled with an almost complete lack of secondary and higher-order veins, collapsing the mechanism that normally governs vein formation. This phenotype is similar to that of the *monopteros* mutant, which is defective in both PAT (Przemeck *et al.*, 1996) and response to auxin (Mattsson *et al.*, 2003). Other mutants showing incomplete secondary strand formation in cotyledons or leaves also have disrupted auxin response maxima (Cnops *et al.*, 2006; Carland *et al.*, 2010). Hence, severe ectopic procambial cell divisions may have disrupted auxin transport and/or signalling to the extent that vein formation no longer occurred. The fact that these leaves could form a primary vein but secondary

vein formation was severely disrupted, and both processes require PIN1-mediated auxin flow inwards from the epidermis (Scarpella *et al.*, 2006; Wenzel *et al.*, 2007), suggests that the feedback loop between secondary strand formation and auxin flow was more susceptible to disruptions in cell division. The *parallel1* mutant, which has parallel-like leaf venation running along the distal/proximal axis into the petiole, lacks marginal auxin response domains (Petricka and Nelson, 2007) that are essential for secondary strand loop formation and connection to the midvein (Scarpella *et al.*, 2006; Wenzel *et al.*, 2007). The multiple parallel strands in the midvein region, incomplete secondary strand formation, and disrupted auxin response maxima in severe Q0990>>C4 phenotypes also implicate essential feedback loops between procambial cell divisions and marginal auxin sources.

Massive vascular overgrowth involves independent cell division and ATI pathways

The combination of ectopic ground cell divisions and ATI, again analogous to the construction of a double mutant, resulted in an increased degree of vascular overproduction compared to the single treatments. This strong synergistic interaction implies that ectopic ground cell divisions and ATI interfered with formation of a normal venation pattern via separate pathways.

It is possible to explain the above results at the tissue level. ATI results in an increased number of vascular cells (Mattsson *et al.*, 1999). Thus, the similarities and epistatic-like relationship between ectopic procambial cell divisions and ATI can be explained by them acting on the same tissue. Similarly, the synergistic effects of combining ectopic ground cell divisions and ATI can be explained by them acting on both ground and procambial tissues. It appears unlikely though that increasing the number of ground cells alone would result in such a strong synergistic effect on leaf vascularization in combination with ATI. One potential explanation is that ectopic ground cell divisions resulted in enhanced auxin response either by increasing local auxin production or by increasing cellular perception of auxin, which in combination with ATI increased the available auxin and triggered formation of ectopic veins. Indeed, ectopic cell divisions in the ground cell tissues did result in ectopic expression of the *DR5::GUS* auxin response marker in cotyledons and young leaf primordia (Fig. 10L–O). However, ectopic procambial divisions also resulted in widespread *DR5::GUS* expression, suggesting that auxin was not the only ground cell factor behind the synergy. It is speculated that ectopic ground cell divisions may continuously provide new cells without fate determination restrictions, allowing a much larger population of cells to respond to the ATI-induced accumulation of auxin in developing leaves (Fig. 12). Regardless of the nature of the factor behind it, the synergistic effect suggests that differences in the regulation of ground cell division activity and auxin production or transport may result in different vein densities observed in plant species.

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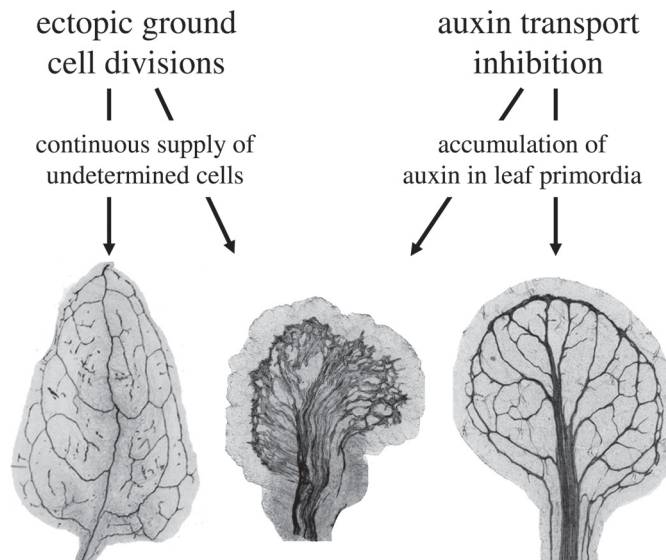


Fig. 12. Interpretation of the synergistic effect of combining ectopic ground cell divisions and auxin transport inhibition on leaf vein development. Auxin transport inhibition leads to overproduction of veins in *Arabidopsis* leaves (right leaf; Mattsson *et al.*, 1999, Sieburth *et al.*, 1999), probably as a consequence of accumulation of auxin in developing leaves as indicated by the *DR5::GUS* auxin response marker (Mattsson *et al.*, 2003). On its own, ectopic ground cell divisions in developing J0571>>C4 leaves result primarily in fragmentation of higher-order veins (left leaf). When combined, ectopic cell divisions and auxin transport inhibition leads to a phenotype not seen in any of the single treatments: a massive vascularization of the leaf blade (centre leaf). We hypothesize that the vascular overproduction in auxin transport-inhibited leaves is limited by simultaneous differentiation of ground cells into mesophyll cells. Ectopic ground cell divisions may negate this effect by providing undifferentiated ground cells that respond to accumulated auxin by differentiation into vascular cells.

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